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Key points

- Both Notch1 and Notch2 receptors are involved in pre-HSC maturation.
- Developing HSCs become Notch independent by the end of their maturation in the AGM region.

Abstract

The first definitive haematopoietic stem cells (dHSCs) in the mouse emerge in the dorsal aorta of the embryonic day (E)10.5-11 aorta-gonad-mesonephros (AGM) region. Notch signalling is essential for early HSC development but is dispensable for the maintenance of adult bone marrow HSCs. How Notch signalling regulates HSCs formation in the embryo is poorly understood. We demonstrate here that Notch signalling is active in E10.5 HSC precursors and involves both Notch1 and Notch2 receptors, but is gradually down-regulated while they progress towards dHSCs at E11.5. This down-regulation is accompanied by gradual functional loss of Notch dependency. Thus, as early as at final steps in the AGM region, HSCs begin acquiring the Notch independency characteristic of adult bone marrow HSCs as part of the maturation programme. Our data indicate that fine stage-dependent tuning of Notch signalling may be required for the generation of definitive HSCs from pluripotent cells.

Introduction

In the mouse embryo the first definitive haematopoietic stem cells (dHSCs), capable of long-term multilineage engraftment in the irradiated adult recipient, emerge in the floor of the dorsal aorta within the AGM region around late E10.5-E11(1-4). HSC development is closely linked to the appearance of intra-aortic haematopoietic cell clusters observed in various vertebrate species, including humans(5-13). Co-expression of endothelial and haematopoietic markers and transcription factors in cluster cells suggests emergence of HSCs and progenitor cells from the underlying haematogenic endothelium(13-17) through a Runx1 dependent process(18-23). Recent observations indicate that the emergence of HSCs involves expansion and gradual maturation of embryonic precursors, termed pre-HSCs, which express an endothelial marker VE-Cadherin (VC) and sequentially upregulate haematopoietic markers such as CD41, CD43 and CD45. Pro-HSCs ($VC^{+}CD45^{-}CD41^{+}CD43^{-}$) detected in E9.5 embryos mature into pre-HSCs Type I ($VC^{+}CD45^{-}CD41^{+}CD43^{+}$) in E10.5 AGM and then into pre-HSCs Type II ($VC^{+}CD45^{+}CD41^{+}CD43^{+}$) which are mainly present at E11.5(24-29). In contrast to dHSCs, these precursors are not detectable by direct transplantations into adult irradiated recipients. A maturation step in an embryonic or neonatal environment is needed to allow them to develop into transplantable dHSCs(24-27).

The Notch pathway is involved in numerous biological processes such as cell fate decisions, stem cell homeostasis, proliferation and apoptosis(30, 31). Interactions of Notch receptors with ligands (in mammals, Notch1-4 and Jag1-2, Dll1, 4, respectively) release the Notch intracellular domain, which, through collaboration with the RBP-Jκ transcription factor, activates Notch targets such as transcriptional repressor Hes1(32). Notch plays an important role in embryonic HSC development(33-35) but is dispensable for adult bone marrow HSCs(36, 37). Notch1 mutant ES cells fail to contribute to adult haematopoiesis, suggesting its cell-autonomous role in HSC specification(38). Notch signalling is required for specification of the haematogenic endothelium in the lateral plate mesoderm(39-41) and for establishing arterial identity of the endothelium, closely related to the haematopoietic specification(33, 42-46). Mouse Notch1, Jag1 or RBP-Jκ mutants are embryonic lethal and exhibit severely impaired haematopoiesis concurrent with expansion of the aortic endothelial cell

population, suggesting regulation of the haematogenic endothelium fate by Notch1-Jag1 signalling(33-35). Notch2 knockouts show no obvious haematopoietic defects(33) and Notch3 and Notch4 knockouts are viable, indicating their non-essential role in HSC development(43, 47). The requirement for Notch in the endothelial-haematopoietic transition is conserved in zebrafish(19, 48-51), where Notch1 acts through activation of and co-operation with important transcription factors such as Gata2, Runx1, Scl, Foxc2 and Hes1/5(34, 48, 50-54). Although Notch is essential for early HSC development, exact stage-specific requirements for this signalling pathway in this multi-step maturation process remain unclear.

Here we show that although Notch signalling is active in and critical for pre-HSC development, down-regulation of Notch activity during transition from the pre-HSC Type I to the Type II stage is essential for this process. However, Notch signalling is largely dispensable for the next step of maturation of pre-HSCs Type II into dHSCs in the AGM region. Although Notch1 is the dominant Notch receptor player, Notch2 also contributes to pre-HSC development. Thus, consistently with the acquisition of the adult status, developing HSCs in the AGM region gain Notch independency, which is a hallmark of adult bone marrow HSCs(36).

Materials and Methods

Mice

Wild type and transgenic mouse lines (all C57BL/6, CD45.2/2) used were: 1) a pHes1-d2EGFP reporter of Hes1 expression(55), 2) Rosa^{CreERT2} (from L. Grotewold and A. Smith), 3) sGFP where GFP is expressed upon Cre-mediated activation(56) and 4) floxed RBP-Jκ(37). The following primers were used for genotyping by PCR: 1) RBP-Jκ as in(57); 2) Rosa^{CreERT2}: F1-AAAGTCGCTCTGAGTTGTTAT, R1-GGAGCGGGAGAAATGGATATG, R2-CATCAAGGAAACCCTGGACTACTG (wild type allele=582bp, Rosa^{CreERT2} allele=249bp); 3) sGFP: F1-AAGTTCATCTGCACCACCG, R1: TCCTTGAAGAAGATGGTGCG (173bp); 4) Hes1-GFP: F1- TCACACAGGATCTGGAGCTG, R1- GAACTTCAGGGTCAGCTTGC (250bp). Mice were housed and bred in animal facilities at the University of Edinburgh, in compliance with the Home

Office regulations. All experiments with animals were conducted under a Home Office UK Project License and approved by University of Edinburgh Ethical Review Committee.

Cultures

Reaggregates and explants were cultured at the liquid-gas interface. Pre-HSC populations sorted from one AGM region were co-aggregated with 10^5 OP9 cells and cultured on filter for 5 days in presence of cytokines (IL-3+SCF+Flt3 ligand; PeproTech) as previously described(25, 26). After culture, single-cell suspensions prepared by dispase/collagenase digestion were used for CFU-C and transplantation assays and FACS analysis(26).

Cre-mediated recombination of RBP-J^{flox} allele was induced by addition of 4-hydroxytamoxifen (4-OHT) (5μM) (Sigma) to cell suspensions for 2h at 37°C before culture.

For blocking Notch, freshly isolated AGM cell suspensions were incubated before culture with DAPT (Calbiochem), or Notch1 and Notch2 blocking antibodies (anti-NRR1 and anti-NRR2, Genentech) for at least 20mn at 37°C. DAPT (50μM) or blocking antibodies (10μg/ml) were also added to the medium at the beginning of the culture.

Transplantations

Embryonic single-cell suspensions (CD45.2/2) were injected into adult recipients (CD45.1/2) along with 20,000 CD45.1/1 bone marrow carrier cells. Recipients were irradiated by split dose (600 + 550 rad with 3h interval) of γ-irradiation. The cell numbers of a particular population are expressed in doses, defined as embryo equivalent (ee), which corresponds to the number of given cells in one AGM region (for example, 0.2ee is equal to 20% of a given cell population in one AGM region). Donor-derived chimerism was monitored in blood at 6 and 14 weeks after transplantation by FACS. Peripheral blood cells treated with PharmLyse were stained with anti-CD16/32, anti-CD45.1-APC (clone A20), and anti-CD45.2-PE (clone 104) antibodies (eBioscience). Different groups of repopulated mice were compared using Mann-Whitney statistical tests.

Flow cytometry

Flow cytometry was performed using FACS Calibur and Fortessa (analysis) and FACS Aria II or FACS Aria Fusion, using FACS Diva software (sorting). Data were analyzed in FlowJo software

(TreeStar). The antibodies used are listed in Table S1. Cell viability was assessed using 7-amino-actinomycin D (7-AAD) or ethidium monoazide (EMA). Gates were set using fluorescence minus one (FMO) controls. All stainings except for RBP-Jκ were performed on live cells. For intra-cellular RBP-Jκ staining, cells were fixed in 2% PFA for 10 min at 4°C and incubated for 30min in 50% FCS, 0.4% Tween 20 before antibody staining.

Haematopoeitic colony assay

Fresh or cultured live cells were seeded into methylcellulose supplemented with cytokines (M3434; StemCell Technologies) for 8-12 days to assess the presence of myeloid colony-forming cells (CFU-Cs). To determine the genotype of RBP-J^{CKO} dHSCs, 20,000- 40,000 cells isolated from recipient BM were seeded into methylcellulose. After culture colonies were individually picked and genotyped to detect RBP-J^{flox} and RBP-J^{Δ} alleles.

Dll1 doxycycline inducible OP9 cell line (OP9-Dll1)

Delta-like1 (Dll1) cDNA was cloned into a doxycycline inducible bicistronic expression vector pPBhCMV1-cHA-IRESVenuspA (gift from H. Niwa(58)) that allowed both Dll1 and Venus to be expressed upon induction. 100,000 OP9 cells were electroporated with this construct using NEON transfecting system (Invitrogen). The next day the electroporated cells were cultured in the presence of $1\mu g \cdot mL^{-1}$ doxycycline (Clontech). The cells which showed no background Venus expression were sorted and used for co-aggregation experiments. Before co-aggregation with AGM cells, OP9-Dll1 cells were pre-cultured with $1\mu g \cdot mL^{-1}$ doxycycline for 24h. Co-aggregates were cultured in the presence of doxycycline for 5 days.

qRT-PCR

Total RNA was isolated from AGM and OP9 with Qiagen RNeasy microkit or minikit (QIAGEN) respectively and cDNA prepared using SuperScriptVILO cDNA Synthesis (Invitrogen). The qRT-PCR was performed using the Light Cycler 480 SYBR Green I Master Mix (Roche). For small number of cells, total RNA was isolated from 200 cells directly FACS sorted and cDNA prepared using CellsDirect (Invitrogen). qRT-PCR was performed using a universal probe library (UPL) and Lightcycler 480 probes mastermix kit (Roche) in duplicates. Expression values were normalized

against the TATA-binding protein (TBP) and standard errors were calculated and plotted using Prism6 software (GraphPad). Primer sequences are available on Table S2.

Results

Notch signals in pre-HSCs and dHSCs

To analyze Notch signalling in the developing HSC lineage, we first examined expression of Notch receptors. QRT-PCR showed that both Notch1 and Notch2 are expressed in phenotypically defined pre-HSC Type I ($VC^+CD45^-CD43^+$) and Type II (VC^+CD45^+) populations in the E11.5 AGM region, whereas Notch3 and Notch4 are expressed at lower, sometimes negligible, levels (Figure 1A). Accordingly, immunophenotyping by flow cytometry showed that Notch1 is highly expressed in most endothelial ($VC^+CD45^-CD41^-$) cells (>90%) and in all pre-HSCs Type I ($VC^+CD45^-CD41^+$) (Figure 1B). Notch1 expression then decreases in pre-HSCs type II (VC^+CD45^+) population so that 30% of them become Notch1-negative (Figure 1B). Conversely, while a minority of endothelial cells and pre-HSCs Type I express Notch2 (16.2% and 22% respectively), more than 70% of pre-HSCs type II become Notch2 positive (Figure 1B, Table S3). We then investigated whether functional pre-HSCs express Notch1 and Notch2 by using the OP9 co-culture system, which allows pre-HSCs to mature into detectable HSCs(24, 25). Functional analysis using sorted Notch1 positive and Notch1 negative cells from E11.5 AGM region followed by co-aggregation with OP9 cells and transplantation into irradiated recipients confirmed that Notch1 is expressed in all pre-HSCs (both Type I and Type II), since only $Notch1^+$ cells were able to generate dHSCs (Figure 1C). Similar functional tests showed that some pre-HSCs Type II also express Notch2 (Figure 1D, Figure S1A-B). Direct transplantations showed that dHSCs from E11.5 AGM region and E12.5 foetal liver can be found in both $Notch1^{low/high}$ and $Notch2^{low/high}$ fractions (Figure S1C-D).

To analyse Notch activity in the developing HSC lineage, we used reporter mice in which a destabilized green fluorescence protein (GFP) is driven by Hes1 promoter(55). We found that Hes1-GFP is expressed in subsets of the endothelial population and of phenotypically defined pre-HSC Type I and Type II populations in the E11.5 AGM region (Figure 2A). QRT-PCR analysis showed quantitative correlation between *Hes1* and GFP transcript levels both in pre-HSC Type I and Type II

populations (Figure S2A). Hes1-GFP$^+$ pre-HSCs showed an enrichment in expression of other Notch target genes, such as Hey1 and Hey2 (Figure S2A). As expected, addition of the Notch/γ-secretase inhibitor, DAPT, efficiently down-regulated Hes1-GFP in both endothelial and pre-HSC populations within 24 hours (Figure S2B, unpublished data). Conversely, addition of doxycycline to co-aggregates with inducible OP9-Dll1 cells (Figure S3A) elevated Hes1-GFP expression significantly in pre-HSCs Type I and to a lesser extent in pre-HSCs Type II (Figure 4A-B). Although Jag1, a weak inducer of Notch activity (59), is expressed in OP9-WT cells (Figure S3B), this was not sufficient to maintain Hes1-GFP expression in pre-HSCs (Figure 4A-B), indicating that this GFP reporter may not reveal weak Notch signalling.

We then functionally tested the status of Notch activity in pre-HSCs. Pre-HSCs Type I (VC$^+$CD45$^-$) prevail in E10.5 embryos and are gradually replaced by pre-HSCs Type II (VC$^+$CD45$^+$) at E11.5(25). Phenotypically, we observed an increase of the GFP subset during HSC maturation (Figure 2A). However functional analysis of sorted GFP+ and GFP- fractions by culture and transplantations showed that pre-HSCs Type I (both at E10.5 and E11.5) were mainly Hes1-GFP$^+$, whereas pre-HSCs Type II resided both in GFP$^-$ and GFP$^+$ fractions (Figure 2B).

The analysis of mature dHSCs sorted from freshly isolated AGM regions one day later at E12.5 showed that they also reside both in the GFP$^-$ and GFP$^+$ fractions (Figure 2C, Figure S4A). The foetal liver HSC population, which consists of mature dHSCs(29) and is defined by SLAM markers(60), was phenotypically predominantly Hes1-GFP+ (Figure 2D) as shown previously(61). However, functional transplantations showed that true dHSCs in E12.5-E14.5 foetal livers reside only in Hes1-GFP^{neg} and Hes1-GFP^{low} fractions, with a tendency towards negative fractions (Figure 2E, Figure S4B and unpublished data). Thus, although Notch signalling is active in all pre-HSCs at E10.5, it is down-regulated in the HSC lineage during further development.

Attenuation of Notch dependency during pre-HSC maturation

Expression analysis of Notch receptors and Hes1-GFP suggested that Notch signalling is functionally involved in pre-HSC maturation. To test this, we first blocked Notch activity by adding DAPT/γ-

secretase inhibitor to AGM explant cultures and assayed the outcome of dHSCs by the long-term repopulation assay (Figure 3A). While addition of DAPT to E10.5 AGM explant cultures almost fully blocked HSC development, the production of dHSCs by E11.5 explants was less affected as most of recipients were repopulated, albeit at significantly lower levels compared to untreated controls (Figure 3A). Accordingly, maturation of purified pre-HSCs Type II from E11.5 embryos was only partially affected by DAPT (Figure 3B). Altogether these data suggest that while most pre-HSCs are sensitive to Notch blockade at E10.5, at later stages they do not require Notch for maturation into dHSC. Meanwhile, in keeping with previous reports(33), production of myeloid progenitors (CFU-C) from E10.5 AGM culture was not significantly affected by DAPT treatment (Figure 3C).

Since DAPT can affect other molecular pathways mediated by γ-secretase, we genetically ablated RBP-Jκ in compound Rbp-Jκ$^{flox/flox}$::Rosa^{CreERT2}::sGFP embryos, hereafter referred to as RBP-J^{CKO}(37, 56). Acute RBP-Jκ ablation was induced in E10.5 AGM cell suspension using 2 hours incubation with 4-hydroxytamoxifen (4-OHT) and after reaggregation and 5 days culture, the generation of dHSCs and CFU-C were assessed functionally (Figure 3E-F). Concurrent Cre-mediated activation of GFP expression upon tamoxifen induction was used as a surrogate marker for Cre-mediated deletion of RBP-Jκ and for tracing donor-derived haematopoietic contribution upon transplantation (Figure S5A).

We found that CFU-Cs were not affected after deletion of RBP-Jκ but expected disruption of HSC development. To our surprise, in 9 out of 10 tested recipients, transplantation of induced RBP-J^{CKO} cells showed GFP positive long-term engraftment comparable with control transplants of wild-type and RBP-J^{Het} cells (RBP-J$^{flox/+}$::Rosa^{CreERT2}::sGFP), although T cell development was blocked at the double-negative stage (CD4^{-}CD8^{-}CD44^{+}) as described previously for Notch deletion in adult bone marrow HSCs(62) (Figure 3E, Figure S5B). At 4 months post transplantation, bone marrow of these recipients contained mainly mutant myeloid progenitors that confirmed repopulation with RBP-J$^{\Delta/\Delta}$ HSCs (Figure S5C). Only one out of 10 recipient transplanted with induced RBP-J^{CKO} cells showed normal T-cell differentiation indicating that in this case repopulation derived from HSCs that escaped deletion (Figure S5C).

Since this result is in apparent discrepancy with DAPT treatment experiments at E10.5 (Figure 3A), we analyzed the clearance of RBP-Jκ protein and found its presence at high levels 24h after induction of genetic ablation (Figure 3G). Only 36h after induction of deletion did RBP-Jκ protein completely disappears (data not shown). We reasoned that the persistence of RBP-Jκ protein during the first day of culture may ensure support of HSC development until the stage at which they become independent of Notch signalling. We tested this hypothesis by adding DAPT with a 24-hours delay and found that in this case the production of dHSCs by pre-HSCs type II indeed, was not affected (compare Figure 3B and 3D). Thus, pre-HSCs become Notch independent shortly before becoming mature dHSCs. Notably, the kinetics of loss of Notch signalling corroborates this conclusion: indeed after 24 hours in culture, pre-HSCs Type II, which initially express Hes1-GFP (Figure 2A), down-regulated GFP (Figure 4B). Meanwhile, DAPT treatment of pre-HSCs Type I with 24-hours delay still negatively affected dHSC maturation (Figure S6), indicating that by that time the Notch independent stage has not yet been reached (Hes1-GFP downregulation observed in this case (Figure 4A) is likely due to insufficient sensitivity of the reporter).

Thus, although at E10.5 pre-HSC development depends on active Notch signalling based on the DAPT treatment experiments (Figure 3A), by E11.5 at least some pre-HSCs down-regulate it (Figure 2B) and their maturation into dHSCs no longer requires Notch (Figure 3A, B, D, E).

Down-regulation of Notch signalling is required for the pre-HSC Type I to Type II transition

Although Notch signalling is down-regulated during HSC maturation and eventually becomes dispensable, it is not clear whether this down-regulation is a necessary step for their development. To test this, we co-aggregated Hes1-GFP^{+} pre-HSCs with Dox-inducible OP9-Dll1 in order to maintain Notch signalling during HSC maturation. In these experiments, we enriched pre-HSCs using positive CD43, cKit, Sca1 and lin-negative markers(24) (data not shown).

Upon doxycycline induction, Dll1 was upregulated in the inducible OP9 cell line (Figure S3A) and, accordingly, Hes1-GFP expression in pre-HSCs was maintained in culture, in contrast to control conditions with un-manipulated OP9 (Figure 4A-B). We found that such an enforced Notch

activity significantly impaired maturation of pre-HSCs Type I but not pre-HSCs Type II (Figure 4C), suggesting that Notch down-regulation is critically important for pre-HSC Type I to Type II transition but not thereafter.

Both Notch1 and Notch2 activity are involved in pre-HSC maturation

We have shown that both Notch1 and Notch2 are expressed in pre-HSCs (Figure 1). We therefore tested whether Notch1 and Notch2 are functionally involved in pre-HSC development using highly specific blocking antibodies, anti-NRR1 and anti-NRR2, respectively(63). Treatment with each antibody led to down-regulation of Notch target genes, comparable to DAPT treatment (Figure S7A-B). Since Notch1, but not Notch2, is involved in arterial specification(64), treatment with anti-NRR1 but not anti-NRR2 down-regulated Notch1 itself and CD44, both known as arterial markers(65) (Figure S7C). As expected, in contrast to Notch1-mediated regulation of itself, anti-NRR2 did not reduce Notch2 protein levels (Figure S7D).

In functional experiments, E10.5 AGM (containing predominantly pre-HSCs type I), or E11.5 sorted pre-HSCs enriched for pre-HSC Type II, were cultured in presence of either anti-NRR1 or anti-NRR2 antibodies. Blocking of either Notch1 or Notch2 drastically suppressed HSC development in the E10.5 AGM region in agreement with DAPT treatment (compare Figure 3A and 5A). Similarly, at E11.5, maturation of sorted pre-HSC Type II was inhibited by addition of blocking antibodies, but to a lesser extent than at E10.5, in agreement with DAPT treatment (compare Figure 3B and 5B). Notably, blocking with anti-NRR2 tended to be more effective than blocking with anti-NRR1, which correlates with an increase of Notch2 and decrease of Notch1 expression in pre-HSCs Type II (Figure 1B). As with DAPT treatment or after RBP-Jκ deletion, the development of CFU-Cs was not disrupted (Figure 5C). Thus, in addition to the previously reported role of Notch1, Notch2 also plays a role in HSC development.

Discussion

Various molecular pathways are involved in early HSC development(66). Notch signalling is critically important for early development of the haematopoietic system(67). Mutants for the main

components of Notch pathway lack haematopoietic progenitors and HSCs and showed an excess of endothelial cells(33-35, 68). Several lines of evidence suggest that Notch controls the development of intra-embryonic haematopoiesis by regulating essential transcription factors which are involved in the endothelial-to-haematopoeitic transition(34, 50). However, HSC development occurs through several sequential maturation steps, which involve a hierarchy of precursors (pre-HSCs) fully committed to haematopoietic fate. The role of Notch signalling in this hierarchical developmental process is not clear. Here we used a combination of *in vitro* modelling of HSC development validated by *in vivo* transplantations(24-26) and conditional genetics to study the role of Notch signalling during the pre-HSC Type I → pre-HSC Type II → dHSC transitions.

Recent evidence suggests that Notch signalling is down-regulated during the emergence of the haematopoietic system in the embryo(69). Notch activity is down-regulated in haematopoietic clusters while maintained in the aortic endothelium and expression of Notch target Hes1 decreases during the endothelia-to-haematopoietic transition(52, 70-74). Furthermore, lowering Notch activity is required for suppression of the arterial programme and acquisition of the haematopoietic fate(71, 73). A recent report has also indicated that Notch signalling in HSCs is lower than in the aortic structural endothelium(59).

Using Hes1-GFP reporter mice we show functionally that while Notch signalling is active in pre-HSCs Type I at E10.5, it is gradually down-regulated during further HSC development, initially in some pre-HSCs Type II at E11.5 and subsequently in the foetal liver where the majority of transplantable HSCs become Hes1-GFP$^{-/low}$.

This down-regulation of Notch signalling has a physiological significance since its maintenance at high levels using exogenous Delta1 is detrimental for maturation of pre-HSCs Type I but not at the next stage, pre-HSC Type II. Furthermore, functional blockade of Notch signalling with DAPT or Notch-specific antibodies prevents pre-HSC Type I development, but only moderately affects pre-HSCs Type II. A further 24 hours delay in DAPT treatment of pre-HSCs Type II completely abrogates the blocking effect, indicating complete loss of Notch dependency by the end of HSC maturation. Notch independence is a feature of adult HSCs(36) and therefore our results are consistent with acquisition of the adult state by developing HSCs.

Accordingly, when we induced RBP-Jκ gene ablation, we expected to see an HSC developmental block at E10.5 and to a lesser extent at E11.5. However, we found that even at E10.5, RBP-Jκ deletion did not prevent development of HSCs, which nevertheless showed a block in T-cell differentiation typical for adult bone marrow RBP-Jκ–null HSCs(62). The generation of RBP-Jκ–mutant HSCs at E10.5 seemed to be in disparity with blocking effects of DAPT and Notch antibodies discussed above. However, a subsequent check showed that the RBP-Jκ protein was present in cells 24 hours after induction of the gene deletion. Such a delay in the loss of the RBP-Jκ protein, perhaps due to its stability, was reported previously in muscle cells(75). This must be sufficient for HSCs to pass the stage of Notch dependency in line with results of the 24-hours delay in DAPT treatment as described above.

How can these data be explained in light of previous study reporting that the loss of RBP-Jκ in the foetal liver is detrimental for HSCs(76)? A possible explanation is that in our experiments, development in the foetal liver is circumvented by direct transplantation of AGM derived RBP-Jκ mutant cells into adult bone marrow environment, where, in contrast to foetal liver, HSCs may not require Notch signalling(36). A similar foetal liver stage-specific dependency was previously proposed for α4 integrin involvement in HSC development(77).

To date, amongst the 4 known Notch receptors, only Notch1 has been shown to play a critical role in early embryonic haematopoiesis in the AGM region(33). Although previous *in situ* analysis suggested the presence of Notch1 but not Notch2 around the E10.5 dorsal aorta(34, 72), our flow cytometry analysis showed clear expression of both Notch1 and Notch2 in the developing HSC lineage. In keeping with this, antibody blockade experiments showed that both receptors are involved in pre-HSC maturation. This contrast with Notch2 knockout studies may be explained by the fact that contrary to genetic ablation, anti-Notch2 antibody does not change the level of Notch2 protein in cells, and thus avoids triggering some compensatory mechanisms. Notably, in adults, both Notch1 and Notch2 continue to be expressed by HSCs(61, 78), although signaling in HSCs is suppressed through intrinsic factors(79), possibly to avoid their potential oncogenic transformation(80). Notch1 and

Notch2 involvement is mainly restricted to downstream differentiation of T-cells and megakaryocyte/erythrocyte progenitors, respectively(61, 62).

It was previously reported that the strength of signalling through Notch2 is weaker than through Notch1(81, 82); therefore, reciprocal kinetics of Notch1 and Notch2 may be responsible for attenuation of the overall Notch activity in the HSC lineage during development. The biological significance of variations in strength of Notch signalling has been reported previously. Oscillations of Hes1 regulate maintenance of neural progenitors and various Notch activity levels control the balance between quiescence and proliferation in adult neural stem cells and pancreatic endocrine progenitors(83-85). It has also been proposed that reduction in strength of Notch signalling is required for switching from the endothelial to the haematopoietic fate(52, 71, 74, 86). Here we specifically focused on Notch signalling during dramatic expansion of pre-HSCs in the AGM region(29) which involves both maturation and slow proliferation(26). Whether down-regulation of Notch signalling plays a role in setting a balance between proliferation and maturation in this process, needs to be elucidated in future.

Recently, massive generation of HSCs from embryonic precursors was achieved in co-cultures with engineered AKT-activated endothelial cells that strongly expressed several Notch ligands(87). Although a Notch-mediated mechanism was proposed, it required TGFβ inhibition and might be explained by other than Notch-mediated mechanisms, as discussed by the authors themselves(87).

In conclusion, the HSC lineage during development in the AGM region switches from a Notch-dependent to a Notch-independent stage (Figure 6). Our results reveal a temporal window of strong Notch dependency during the pre-HSC Type I to pre-HSC Type II transition in the E10.5-E11.5 AGM region (Figure 6). Once pre-HSCs pass this stage, they become significantly less dependent on Notch, which is consistent with Notch independence in adult bone marrow HSCs(36, 88). Although Notch1 is dominant, both Notch1 and Notch2 have functional roles in HSC development. Careful stage-specific tuning of Notch signalling may be required when developing conditions for the generation of transplantable HSCs from pluripotent ES/iPS cells for therapeutic applications.

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Figure Legends

Figure 1: Expression of Notch receptors in HSC lineage in AGM region.

A: **Expression levels of Notch receptors** assessed by qRT-PCR in endothelial cells ($VC^+CD45^-CD43^-$), pre-HSCs type I ($VC^+CD45^-CD43^+$) and pre-HSCs type II (VC^+CD45^+) sorted from the E11.5 AGM region; (n=3). Data are mean±s.e.m. *P<0.05, **P<0.01, ns: non-significant, t-test.

B: Expression of Notch1 and Notch2 in HSC lineage. FACS analysis representing Notch1 or Notch2 presence at the surface of endothelial cells **(**$VC^+CD45^-CD41^-$**)**, pre-HSCs Type I ($VC^+CD45^-CD41^+$**)** and pre-HSCs Type II **(**VC^+CD45^+) in E11.5 AGM region (n=3). The graphs on the right indicate MFI ratios between Notch1 or Notch2 and their respective FMO controls during the endothelial-to-pre-HSC transition in 3 independent experiments. Data are mean±s.e.m. *P<0.05, ns: non-significant, t-test.

C: All functional pre-HSCs express Notch1. E11.5 AGM cells were sorted based on Notch1 expression and 2 populations ($Notch1^+$ and $Notch1^-$) were co-aggregated with OP9 cells and cultured for 5 days before transplantation into irradiated mice in order to functionally assess the presence of pre-HSCs (0.5ee/recipient); (n=2); (*: p<0.05, Mann-Whitney U test).

D: **Notch2 is expressed in functional pre-HSCs Type II, but not in pre-HSCs Type I**. E11.5 VC^+CD45^- cells (Type I) and VC^+CD45^+ (Type II) were sorted based on Notch2 expression level and co-aggregated with OP9. After 5 days of culture they were injected into irradiated recipients; (pre-HSC Type I: 1ee/recipient; pre-HSC Type II: 0.1ee/recipient); (n=2); (***: p<0.005, Mann-Whitney U test).

Figure 2: Notch activity decreases during HSC maturation.

A: Expression of Hes1-GFP in endothelial cells ($VC^+CD45^-CD43^-$) (Gate **R1)**, pre-HSCs Type I ($VC^+CD45^-CD43^+$) (Gate **R2)** and pre-HSCs Type II ($VC^+CD45^+CD43^+Sca1^+$) (Gate **R3)** defined by

flow cytometry in E11.5 Hes1-GFP$^+$ AGM region. FMO GFP control (Fluorescence Minus One control) was performed with wild type cells.

B: Pre-HSCs Type I are mainly Hes1-GFP$^+$, while pre-HSCs Type II reside in both GFP$^-$ and GFP$^+$ fraction. VC$^+$CD45$^-$ (Pre-HSCs type I) and VC$^+$CD45$^+$ (pre-HSCs type II) cells were sorted from E10.5 and E11.5 AGM based on Hes1-GFP expression, co-aggregated with OP9 cells and transplanted after culture; (1ee/recipient); (n=3). Levels of engraftment are plotted and number of repopulated versus total number of transplanted mice are shown in brackets; (***: $p<0.005$, Mann-Whitney U test).

C: **AGM dHSCs reside in both Hes1-GFP$^+$ and Hes1-GFP$^-$ populations.** CD45$^+$ cells were sorted from E12.5 AGM based on Hes1-GFP expression and directly transplanted into irradiated mice; (4ee/recipient); (n=2).

D: Expression of Hes1-GFP in E14.5 foetal liver dHSCs, phenotypically defined by Lin$^-$ cKit$^+$Sca1$^+$CD48$^-$CD150$^+$. LSK: Lin$^-$Sca$^+$cKit$^+$. Grey histogram: Hes1-GFP$^-$ control.

E: **Foetal liver HSCs reside in GFP$^{-/low}$ fraction**. LSK populations were sorted based on Hes1-GFP expression from E13.5 and E14.5 foetal liver (FL) and directly transplanted into irradiated mice; (0.2ee/recipient); (n=2).

Figure 3: Notch signalling is required for pre-HSC development.

A: **DAPT treatment completely prevents HSC development in E10.5 AGM, but has a milder effect at E11.5.** E10.5 (n=2) and E11.5 (n=2) explants were cultured for 5 days without cytokines in presence of DMSO or 50μM of DAPT. At the end of the culture the explants were dissociated and injected into irradiated mice; (0.3ee/mouse); (**: $p<0.01$, Mann-Whitney test).

B: **DAPT treatment disrupts pre-HSCs Type II maturation.** E11.5 sorted VC$^+$CD45$^+$ cells (pre-HSC type II) were co-aggregated with OP9. After 5 days of culture with cytokines in presence of DMSO or 50uM of DAPT the reaggregates were dissociated and injected into irradiated mice. (0.2ee/mouse); (n=3); (**: $p<0.01$, Mann-Whitney test).

C: **DAPT treatment does not affect CFU-C development.** E10.5 explants were cultured for 5 days without cytokines in presence of DMSO or 50μM of DAPT. After culture, the development of haematopoietic progenitors (CFU-C) was assessed by performing a colony forming assay. Bars represent the average number of CFU-Cs per ee and standard errors; (n=3).

D: **DAPT does not affect pre-HSC Type II maturation into HSCs when added after 24 hours of culture.** E11.5 sorted Hes1-GFP^{+} pre-HSC type II were cultured for 24h prior addition of DMSO or DAPT. After subsequent 4 days in culture, the cells were injected into irradiated mice; (0.2ee/mouse); (n=2).

E: Conditional deletion of RBP-Jκ in E10.5 AGM region. E10.5 RBP-J^{CKO}, RBP-J^{Het} and wild type (wt) AGM cells were dissociated, treated individually with 4-OHT for 2 hours and then cultured as reaggregates without OP9 and without cytokines for 5 days before transplantation. The red triangles represent the recipients whose bone marrow contained RBP-J $^{\Delta / \Delta}$ dHSCs and showed T-cell phenotype; the blue triangle represents the mouse repopulated with RBP-J$^{flox/\Delta}$ dHSCs (normal T-cell development); black triangles represent recipients whose bone marrow was not analysed further; (0.2ee/mouse).

F: Numbers of CFU-Cs per ee in E10.5 RBP-J^{CKO} (CKO), RBP-J^{Het} (Het) and WT AGM after culture (4, 3 and 4 embryos respectively; standard errors are shown).

G: Presence of RBP-Jκ protein after induction of the deletion. E10.5 RBP-J^{CKO} AGM was dissociated and divided into 2 parts, one was treated with 4-OHT and the other with methanol (4-OHT vehicle) for 2 hours at 37^{o}C. 24 hours after induction, the presence of RBP-Jκ protein was analysed by flow cytometry. The dot plots are representative of 4 different AGMs, gated on live cells (EMA^{-}).

GM: Granulocyte-Macrophage, M: Macrophage, Mast: Mast colonies, BFU-E: Blood Forming Unit-Erythrocyte.

Figure 4: Forced Notch activity blocks Pre-HSC type I maturation.

A, B: Forced activation of Notch activity elevates Hes1-GFP expression in pre-HSCs Type I and Type II. Hes1-GFP^{+} pre-HSC Type I (lin^{-}VC^{+}CD45^{-}CD43^{+}cKit^{+}) and Type II (lin^{-}

$VC^+CD45^+Sca1^+cKit^+$) were sorted from E11.5 AGM and co-aggregated either with OP9-WT (left column) or OP9 expressing DLL1 upon doxycycline addition (right columns). After one day of culture VC^+CD45^+ cells (dot plots) derived from pre-HSCs Type I and Type II were analysed for Hes1-GFP expression (black histograms) and compared to Hes1-GFP negative control cells (grey histograms). The data are representative of 2 independent experiments.

C: **Forced Notch activity prevents pre-HSC Type I maturation.** Hes1-$GFP^+lin^-VC^+CD45^-CD43^+cKit^+$ pre-HSCs Type I and Hes1-$GFP^+lin^-VC^+CD45^+Sca1^+cKit^+$ pre-HSCs Type II were sorted from E11.5 AGM regions and reaggregated with OP9-Dll1. The co-aggregates were cultured with cytokines with or without doxycycline for 5 days before transplantation. (0.1ee/recipient); (n=2); (**: $p<0.01$, Mann-Whitney test).

Figure 5: Blocking Notch1 or Notch2 negatively affects HSC development.

A: Both Notch1 and Notch2 are involved in HSC development in E10.5 AGM region. E10.5 AGM were cultured as reaggregates for 5 days without cytokines in presence of anti-NRR1 (10ug/ml) or anti-NRR2 (10ug/ml) antibodies before transplantation into irradiated mice. (0.3ee/recipient); (n=2); (*: $p<0.05$, Mann-Whitney test).

B: Blocking Notch1 or Notch2 affects pre-HSC Type II maturation. E11.5 pre-HSC Type II (VC^+CD45^+) were co-aggregated with OP9 cells with cytokines and cultured for 5 days with anti-NRR1 (10ug/ml) or anti-NRR2 (10ug/ml) antibodies before transplantation; (0.1ee/recipient); (n=2); (*: $p<0.05$, Mann-Whitney test).

C: Blocking Notch1 or Notch2 does not disrupt CFU-C development. The development of haematopoietic progenitors in presence of Notch blocking antibodies after 5 days in culture, in E10.5 AGM reaggregates was assessed by CFU-C assay. The number of haematopoietic progenitors per 1ee and standard errors are shown (n=2).

GEMM: Granulocyte-Erythrocyte-Macrophage-Megakaryocyte, GM: Granulocyte-Macrophage, M: Macrophage, Mast: Mast, BFU-E: Blood Forming Unit-Erythrocyte.

Figure 6: Model

In the E10.5 AGM region, Hes1-GFP is expressed in all functional pre-HSCs Type I, demonstrating that Notch pathway is activated in these cells. Notch1 is the main receptor at this stage; later on, Notch2 is upregulated during the pre-HSC Type I to pre-HSC Type II transition. Although both Notch1 and Notch2 are expressed in maturing pre-HSCs and dHSCs, Notch activity decreases since some pre-HSCs and dHSCs at E11.5 and E12.5, respectively, become Hes1-GFP^{-}. Functional analysis showed that Notch activity is essential during the first steps of pre-HSCs development. However, the decrease of Notch activity is accompanied by a progressive loss of Notch dependency, as some E11.5 pre-HSCs can complete development in the absence of Notch. In the foetal liver HSCs either fully lack Notch activity or exhibit it at a low level, despite the presence of both Notch1(61, 76) and Notch2 at their surface.

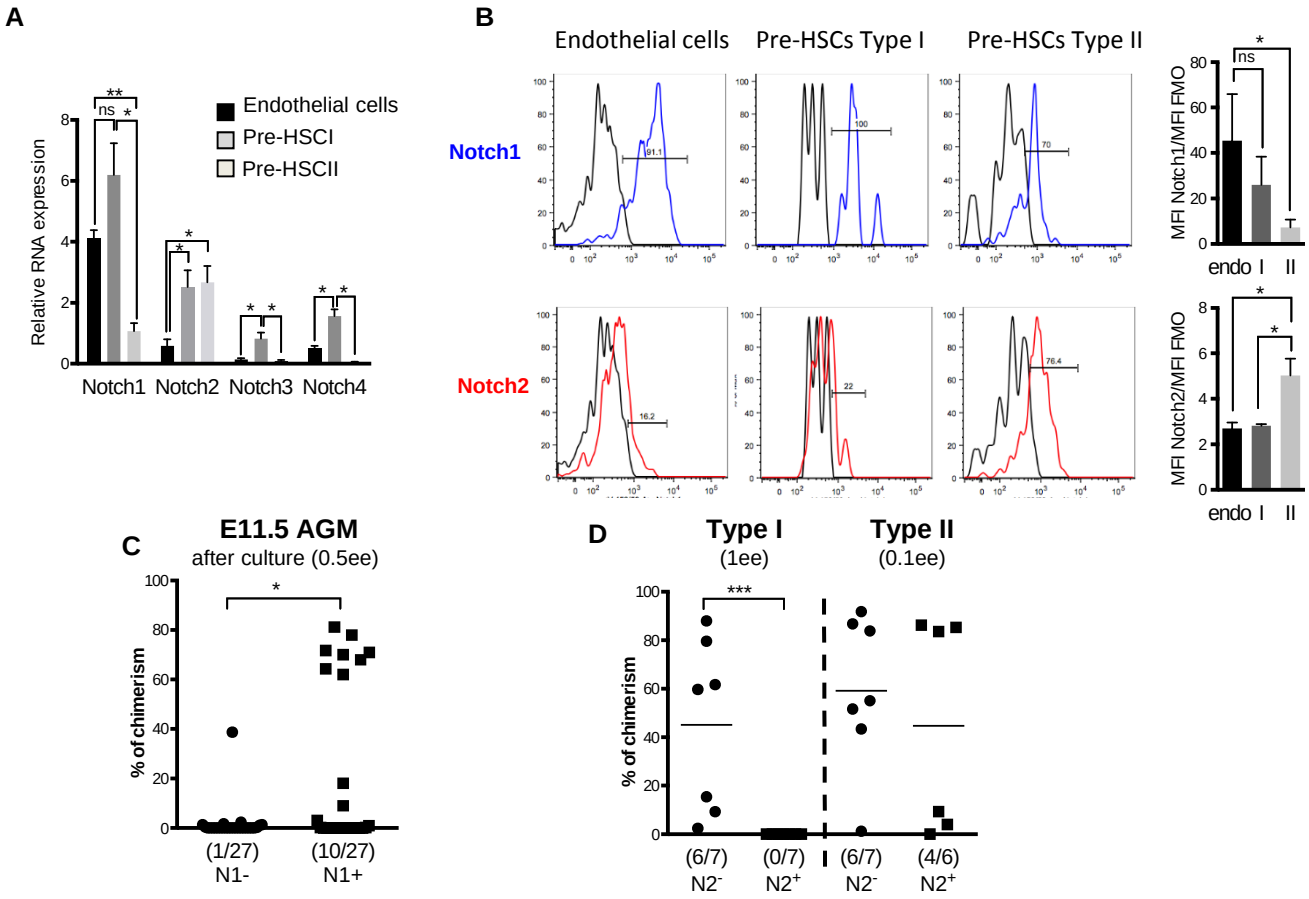


Figure 1: Expression of Notch receptors in HSC lineage in AGM region.

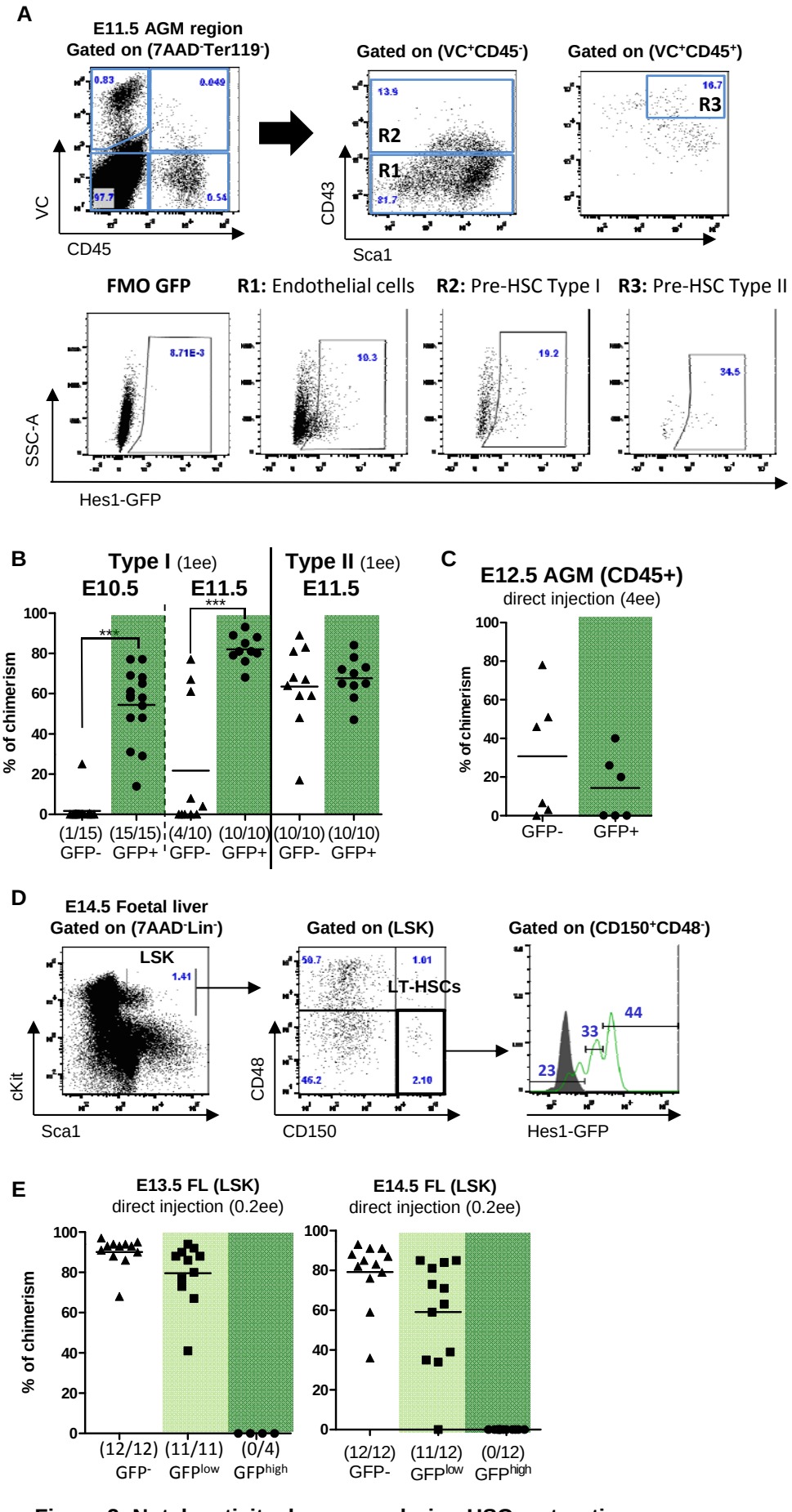


Figure 2: Notch activity decreases during HSC maturation

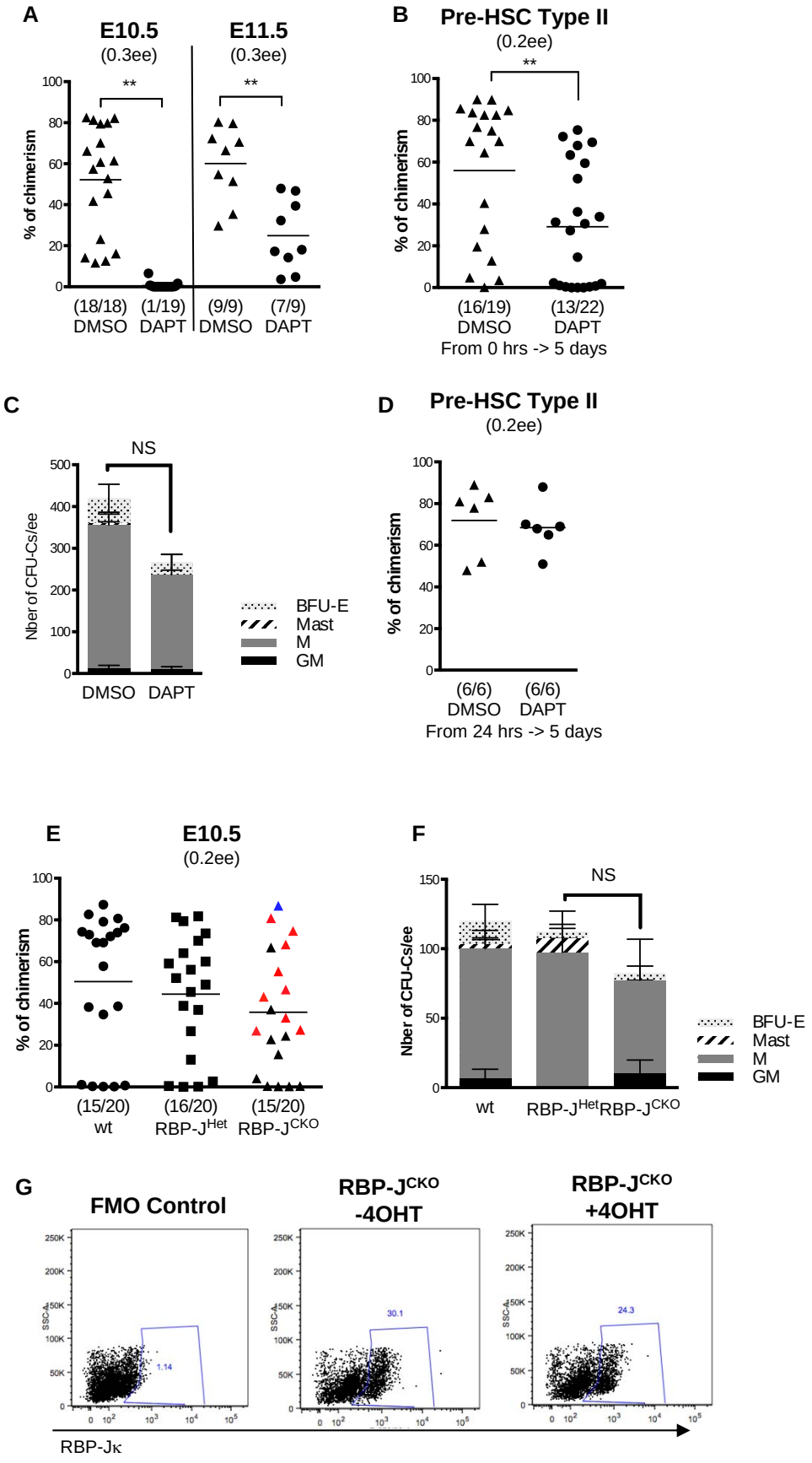
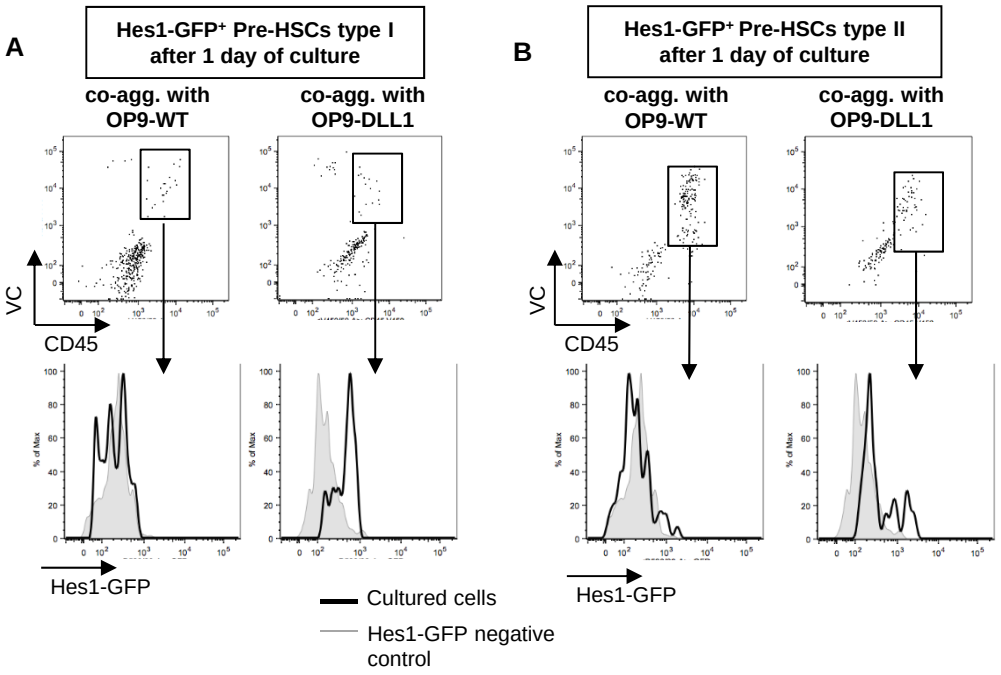


Figure 3: Notch signalling is required for pre-HSC development.



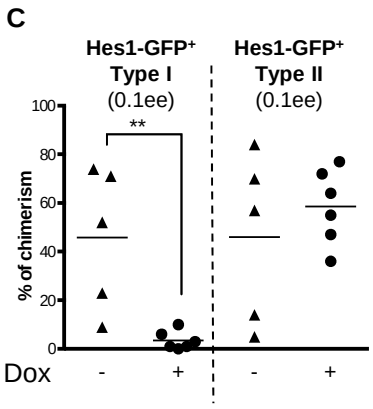


Figure 4: Forced Notch activity blocks Pre-HSC Type I maturation.

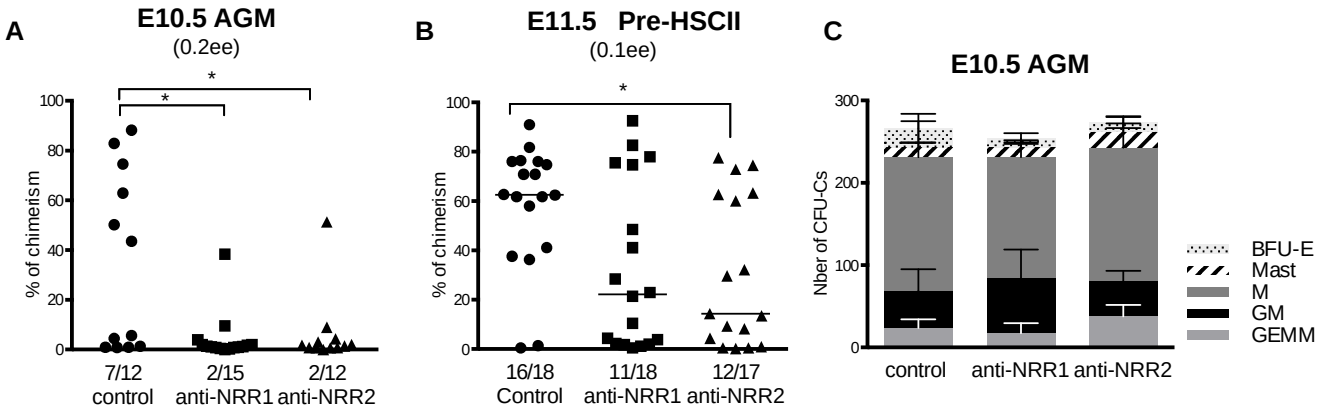


Figure 5: Blocking Notch1 or Notch2 negatively affects HSC development.

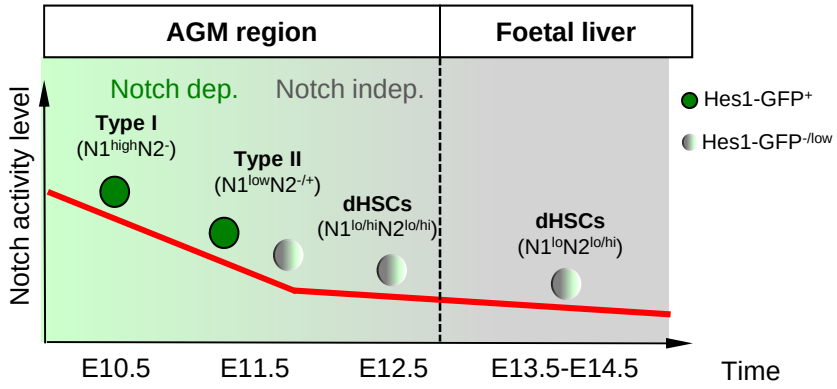


Figure 6: Model

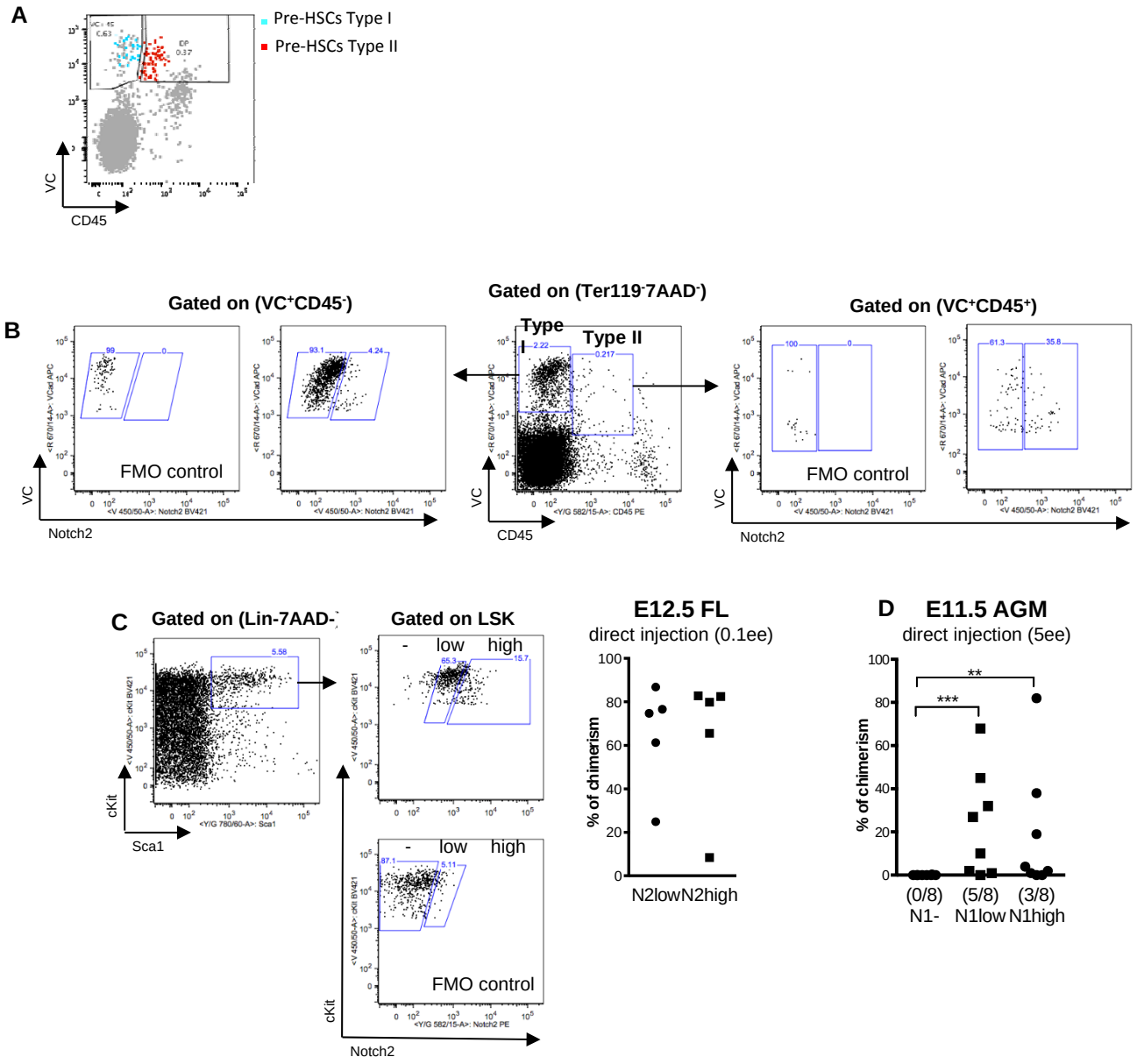


Figure S1: Expression of Notch1 and Notch2 in pre-HSCs/dHSCs.

A: Back-gating strategy showing expression levels in Pre-HSCs Type I (lin- VC+ CD45- CD41+ cKit+) and pre-HSCs Type II (Lin- VC+ CD45+ cKit+ Sca1+) cells. Functional pre-HSC Type II are $CD45^{low}$.

B: Gating strategy to sort pre-HSCs Type I ($VC^{+}CD45^{-}$) and Type II ($VC^{+}CD45^{low}$) based on Notch2 expression.

C: Foetal liver dHSCs reside in $Notch2^{low/high}$ fraction. Gating strategy to sort $Notch2^{low}$ and $Notch2^{high}$ LSK cells from E12.5 foetal liver. Sorted cells were then directly injected into recipient mice; (0.1ee/mouse); (n=1).

D: AGM dHSCs reside in $Notch1^{low/high}$ fraction. E11.5 AGM cells were sorted based on Notch1 expression and directly injected into irradiated mice in order to assess the presence of dHSCs. (5ee/recipient); (n=2).

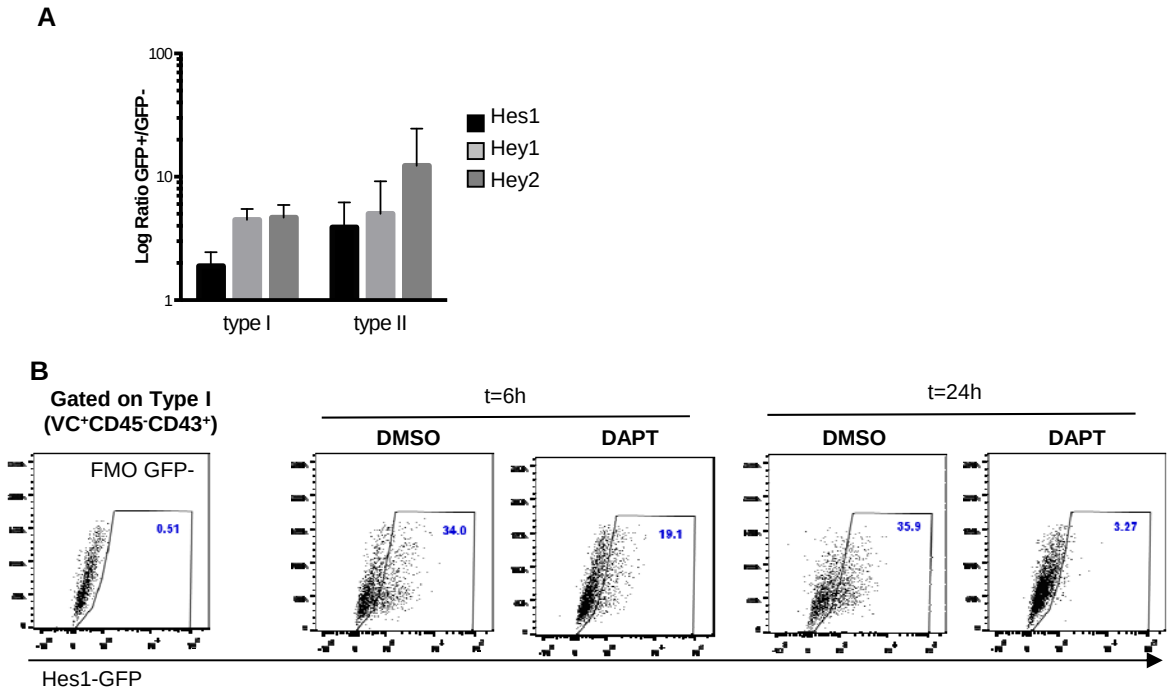
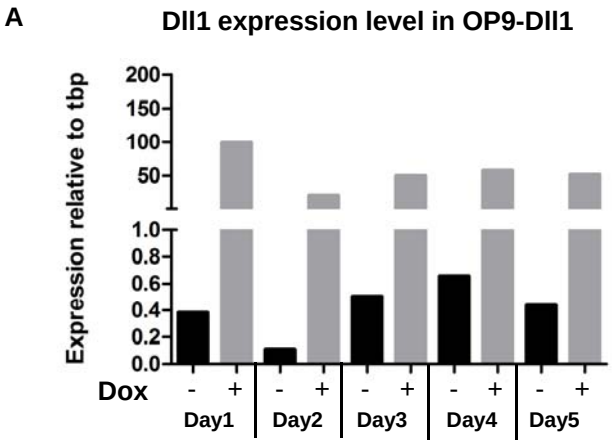


Figure S2: Hes1-GFP is a reliable reporter of Notch activity in pre-HSC/dHSC lineage.
A: Expression level of endogenous Notch target genes Hes1, Hey1 and Hey2 in sorted Hes1-GFP^{-} and Hes1-GFP^{+} pre-HSCs assessed by qRT-PCR, n=3. Data are mean±s.e.m.
B: Expression of Hes1-GFP^{+} in pre-HSCs type I (VC^{+}CD45^{-}CD43^{+}) population after treatment with DAPT for 6h and 24h compared to the DMSO treated control assessed by FACS.



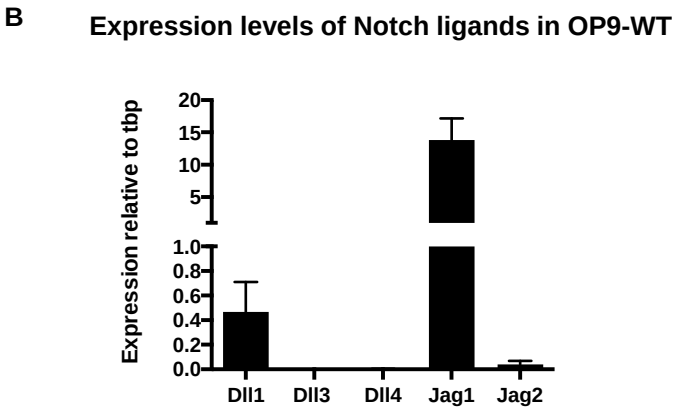


Figure S3: Expression of Notch ligands in OP9-Dll1 and OP9-WT cell lines assessed by qRT-PCR.
A: Expression of Dll1 in OP9-Dll1 cells during 5 days in culture after induction with doxycycline.
B: Expression levels of Notch ligands in OP9-WT cells after 48h in culture (n=4; mean SD).

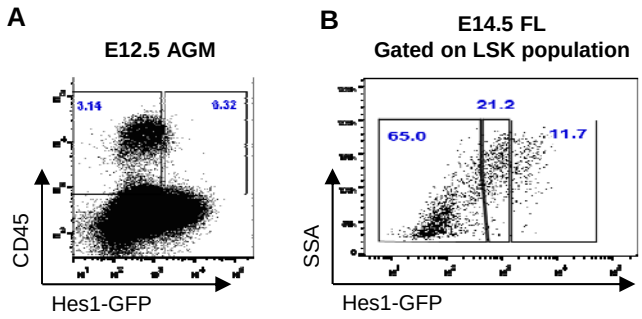
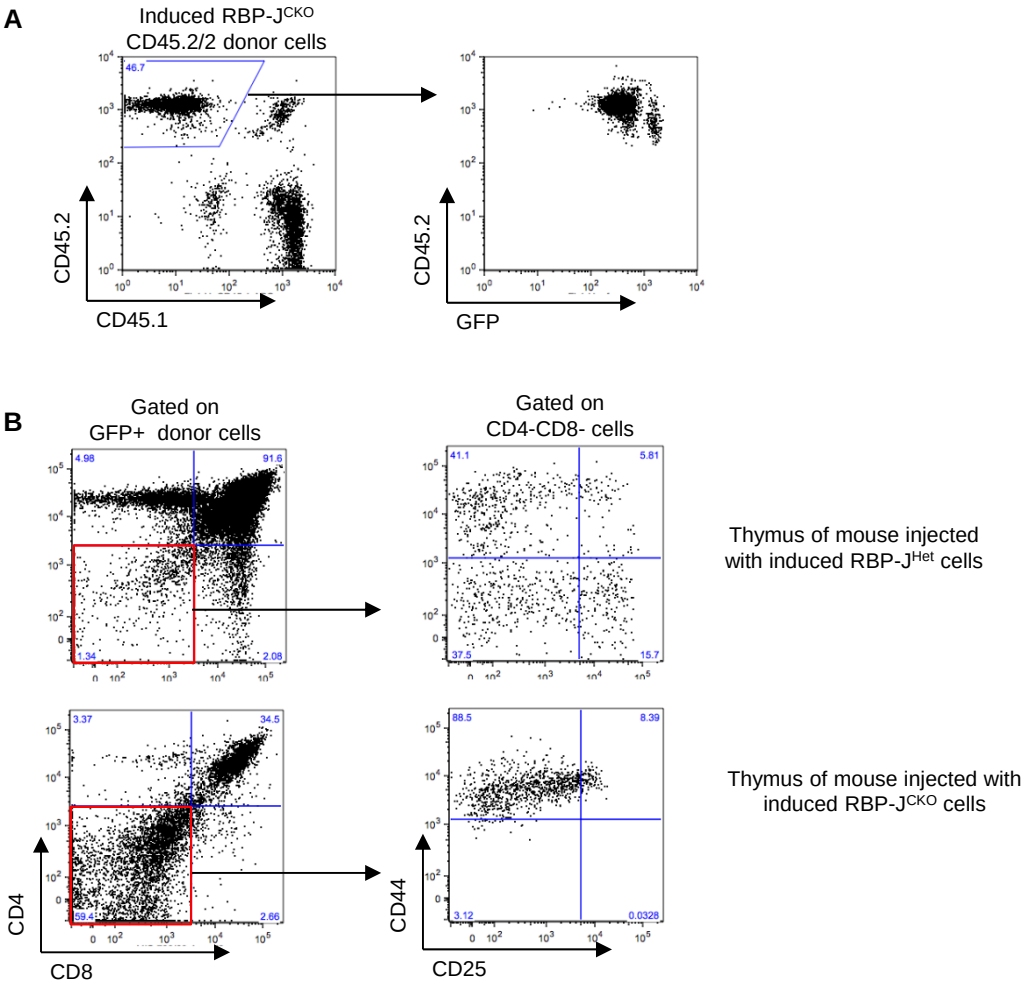


Figure S4: Expression of Hes1-GFP in dHSCs in AGM region and foetal liver.
A: Gating strategy to sort $CD45^{+}$ cells from E12.5 AGM based on Hes1-GFP expression.
B: Gating strategy to sort LSK cells from E14.5 foetal liver based on Hes1-GFP expression.



C

% of repopulation in recipients	Genotype and Number of GFP$^+$ colonies		T cell phenotype
	Flox/Δ	Δ/Δ	
66.8%	1	14	✓
33.2%	0	2	✓
86.8%	2	16	✓
80.2%	24	0	
27.4%	0	7	✓
55.4%	0	5	✓
27%	0	6	✓
74.8%	0	12	✓
43.2%	0	20	✓
46.7%	0	9	✓

Figure S5: Generation of HSCs after deletion of RBP-Jk in the E10.5 AGM region.

A: Dot plot representing the contribution of CD45.2/2 donor induced RBP-J^{CKO} to the host haematopoietic system (CD45.1/2) in the blood of a recipient mouse 16 weeks post-transplantation. All donor cells are GFP$^+$, as a result of Cre-mediated activation of sGFP. Note the presence of CD45.1/1 carrier cells.

B: Analysis of T-cells in thymi of a control recipient (injected with RBP-J^{Het} cells) and recipient injected with induced RBP-J^{CKO} AGM cells. The high proportion of CD4-CD8-CD44+ cells in the thymus of recipient injected with RBP-J^{CKO} AGM cells compared to the control is characteristic of T-cell blockade observed in Notch mutants.

C: Number and genotype of GFP$^+$ haematopoietic colonies obtained after plating bone marrow of 10 recipient mice in CFU-C cultures. All recipients were transplanted with induced RBP-J^{CKO} AGM cultured cells.

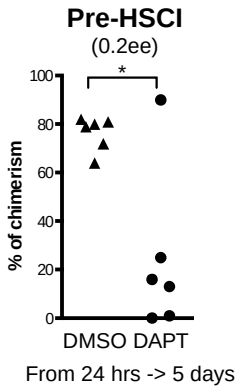


Figure S6: DAPT still affects pre-HSC Type I maturation into HSCs when added after 24 hours of culture. E11.5 sorted Hes1-GFP^{+} pre-HSC type I were cultured for 24h prior to addition of DMSO or DAPT. After subsequent 4 days in culture, the cells were injected into irradiated mice; (0.2ee/mouse); (n=2).

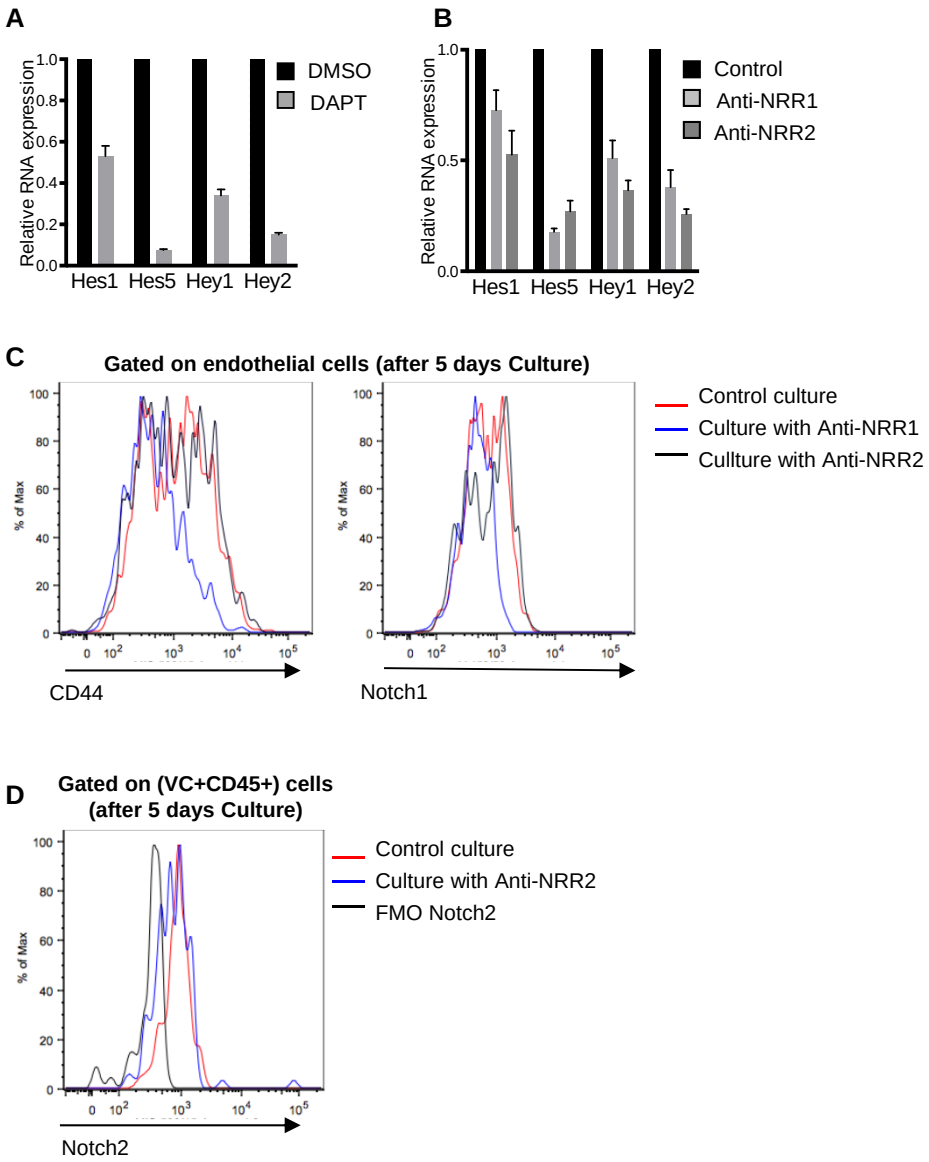


Figure S7: Effect of DAPT and Notch Blocking Antibodies on Notch target genes and arterial markers.

A: Expression levels of Notch target genes in E11.5 AGM explants after 5 days in culture with DMSO or 50uM DAPT; (n=2).

B: Expression levels of Notch target genes in E11.5 AGM reaggregates after 5 days of culture in presence or absence of anti-NRR1 and anti-NNR2 blocking antibodies; (n=2).

C: Expression of CD44 and Notch1 in endothelial cells after treatment with anti-NRR1 or anti-NRR2 blocking antibodies. The plots are representative of 4 experiments.

D: Expression of Notch2 in $VC^{+}CD45^{+}$ cells after treatment with anti-NRR2. The plot is representative of 2 experiments.

Table S1: Antibodies used for flow cytometry

Epitope	Clone	Conjugate
CD45.1	A20	APC
CD45.2	104	PE
CD45	30F-11	PE/V450
cKit	2B8	Bio/APC
Sca1	D7	PeCy7/BV421
CD44	IM7	PE/BV421
CD4	GK1.5	APC
CD8a	53-6.7	Pe-Cy7
CD25	PC61	PE
CD43	eBioR2/60	PE
CD48	HM48-1	PE
CD150	12F12.2	PE-Cy7
Ter119	TER119	PE/PerCp-Cy5.5/Bio
B220	RA3-6B2	PerCp-Cy5.5/Bio
CD3e	145-2c11	PerCp-Cy5.5/Bio
Ve-Cadh	eBioBV13	Al647
Ve-Cadh	11D4.1	Bio
Notch1*	22E5.5	Bio
Notch1	mN1A	PE
Notch2	16F11	PE/Bio
RBP-J*	1F1	Unconjugated

All antibodies were purchased from eBiosciences, BD pharmingen and BioLegend. Streptavidin coupled to AlexaFluor700, PE or BV421 and the secondary antibody chicken anti-Rat Alexa633 were also used. The lineage cocktail was composed of antibodies against CD3e, B220 and Ter119.

*Notch1 antibody (clone 22E5.5) was manufactured in R. Mac Donald's lab.

*RBP-J antibody was obtained from Ascenion.

All the staining were performed on live cells except for RBP-J staining.

Table S2: Primers used for qRT-PCR

Gene	sequence	UPL probe
Notch1	F-ctggaccccatggacatc R-aggatgactgcacacattgc	80
Notch2	F-tgcctgtttgacaactttgagt R-gtggtctgcacagtatttgtcat	6
Notch3	F-agctgggtcctgaggtgat R-agacagagccggttgtcaat	9
Notch4	F-ggacctgcttgcaaccttc R-tgacctccacctcacagagtc	34
Hes1	F- acaccggacaaaccaaagac R- cgcctcttctccatgatagg	99
Hes5	F- ccaaggagaaaaaccgactg R- cttggagttgggctggtg	15
Hey1	F-catgaagagagctcacccaga R-gaacacagagccgaactcaa	17
Hey2	F-gtggggagcgagaacaatta R-gttgtcggtgaattggacct	105
Dll1	F-agcaaacgtgacaccaagtg R-taagtgttggggcggatcttc	2
Dll1	F- gaaggttgctctgtgttctgc R- ccctggcagacagattgg	
Dll3	F- gggggcagctgtagtgaa R- acatcgaagcccgtagaatc	
Dll4	F- cgggaaccttctcactcaac R- ttggatgatgatttggctga	
Jag1	F- gaggcgtcctctgaaaaaca R- acccaagccactgttaagaca	
Jag2	F-ctcctcctgctgctttgtg R-tgtcaggcaggtcccttg	
Tbp	F- ggggagctgtgatgtgaagt R- ccaggaaataattctggctca	

Table S3: MFI of Notch2 expression in endothelial cells, pre-HSCs Type I and Type II compared to FMO controls.

		MFI Notch2	MFI FMO Notch2	ΔMFI
Exp1	Endo cells	559	172	387
	Pre-HSC I	606	212	394
	Pre-HSC II	1012	210	802
Exp2	Endo cells	355	144	211
	Pre-HSC I	676	249	427
	Pre-HSC II	1751	272	1479
Exp3	Endo cells	416	175	241
	Pre-HSC I	671	229	442
	Pre-HSC II	1147	294	853

MFI: Mean Fluorescence Intensity,
FMO: Fluorescence Minus One